

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | | |
|---|--|-----------|---|
| <p>(51) International Patent Classification ⁶ : C12N 15/53, A01H 5/02, 5/00</p> | | <p>A1</p> | <p>(11) International Publication Number: WO 96/36716 (43) International Publication Date: 21 November 1996 (21.11.96)</p> |
| <p>(21) International Application Number: PCT/AU96/00296</p> | | | <p>(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> |
| <p>(22) International Filing Date: 16 May 1996 (16.05.96)</p> | | | |
| <p>(30) Priority Data: PN 2988 16 May 1995 (16.05.95) AU</p> | | | |
| <p>(71) Applicant (<i>for all designated States except US</i>): INTERNATIONAL FLOWER DEVELOPMENTS PTY. LTD. [AU/AU]; 16 Gipps Street, Collingwood, VIC 3066 (AU)</p> | | | <p>Published <i>With international search report.</i></p> |
| <p>(72) Inventor; and (75) Inventor/Applicant (<i>for US only</i>): HOLTON, Timothy, Albert [AU/AU]; Unit 1, 19 Oldis Street, Northcote, VIC 3070 (AU)</p> | | | |
| <p>(74) Agents: HUGHES, E., John, L. et al.; Davies Collison Cave, 1 Little Collins Street, Melbourne, VIC 3000 (AU)</p> | | | |
| <p>(54) Title: TRANSGENIC PLANTS EXHIBITING ALTERED FLOWER COLOUR AND METHODS FOR PRODUCING SAME</p> | | | |
| <p>(57) Abstract</p> <p>The present invention relates generally to novel approaches in generating transgenic plants exhibiting altered flower colour by the introduction of a dihydroflavonol-4-reductase (DFR) which preferably acts on dihydromyricetin (DHM). More particularly, the present invention provides transgenic carnation plants and flowers cut therefrom exhibiting flower colouration not naturally associated with carnation plants. The present invention further contemplates methods for producing transgenic carnation plants with the altered flower colour.</p> | | | |

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|----|--------------------------|----|---------------------------------------|----|--------------------------|
| AM | Armenia | GB | United Kingdom | MW | Malawi |
| AT | Austria | GE | Georgia | MX | Mexico |
| AU | Australia | GN | Guinea | NE | Niger |
| BB | Barbados | GR | Greece | NL | Netherlands |
| BE | Belgium | HU | Hungary | NO | Norway |
| BF | Burkina Faso | IE | Ireland | NZ | New Zealand |
| BG | Bulgaria | IT | Italy | PL | Poland |
| BJ | Benin | JP | Japan | PT | Portugal |
| BR | Brazil | KE | Kenya | RO | Romania |
| BY | Belarus | KG | Kyrgyzstan | RU | Russian Federation |
| CA | Canada | KP | Democratic People's Republic of Korea | SD | Sudan |
| CF | Central African Republic | KR | Republic of Korea | SE | Sweden |
| CG | Congo | KZ | Kazakhstan | SG | Singapore |
| CH | Switzerland | LI | Liechtenstein | SI | Slovenia |
| CI | Côte d'Ivoire | LK | Sri Lanka | SK | Slovakia |
| CM | Cameroon | LR | Liberia | SN | Senegal |
| CN | China | LT | Lithuania | SZ | Swaziland |
| CS | Czechoslovakia | LU | Luxembourg | TD | Chad |
| CZ | Czech Republic | LV | Larvia | TG | Togo |
| DE | Germany | MC | Monaco | TJ | Tajikistan |
| DK | Denmark | MD | Republic of Moldova | TT | Trinidad and Tobago |
| EE | Estonia | MG | Madagascar | UA | Ukraine |
| ES | Spain | ML | Mali | UG | Uganda |
| FI | Finland | MN | Mongolia | US | United States of America |
| FR | France | MR | Mauritania | UZ | Uzbekistan |
| GA | Gabon | | | VN | Viet Nam |

TRANSGENIC PLANTS EXHIBITING ALTERED FLOWER COLOUR AND METHODS FOR PRODUCING SAME

5 The present invention relates generally to novel approaches in generating transgenic plants exhibiting altered flower colour. More particularly, the present invention provides transgenic carnation plants and flowers cut therefrom exhibiting flower colouration not naturally associated with carnation plants. The present invention further contemplates methods for producing transgenic carnation plants with the altered flower colour.

10

Bibliographic details of the publications referred to in this specification are collected at the end of the description. Sequence Identity Numbers (SEQ ID NOS.) for the nucleotide sequences referred to in the specification are defined following the bibliography.

15 Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

20 The rapidly increasing sophistication of recombinant DNA technology is greatly facilitating a broad spectrum of industrial processes from the horticultural to medical and allied health industries. The horticultural and related agricultural industries are particularly benefiting from the advances in recombinant DNA technology.

25 The floriculture industry in particular strives to develop new and different varieties of flowering plants, with improved characteristics ranging from disease and pathogen resistance to altered flower colour. Although classical breeding techniques have been used with some success, this approach has been limited by the constraints of a particular species' gene pool. It is rare, for example, for a single species to have a full spectrum of coloured varieties.

30 Accordingly, substantial effort has been directed towards the use of recombinant DNA

technology to generate transgenic plants exhibiting the desired characteristics.

The development of varieties of the major cutflower species such as carnation plants, for example, having flowers exhibiting a range of colours covering lilac, violet, purple and blue 5 or various shades thereof, would offer a significant opportunity in both the cutflower and ornamental markets.

Flower colour is predominantly due to two types of pigment: flavonoids and carotenoids. Flavonoids contribute to a range of colours from yellow to red to blue. Carotenoids impart 10 an orange or yellow tinge and are commonly the only pigment in yellow or orange flowers. The flavonoid molecules which make the major contribution to flower colour are the anthocyanins which are glycosylated derivatives of cyanidin, delphinidin, petunidin, peonidin, malvidin and pelargonidin, and are localised in the vacuole. The different anthocyanins can produce marked differences in colour. Flower colour is also influenced by co-pigmentation 15 with colourless flavonoids, metal complexation, glycosylation, acylation, methylation and vacuolar pH (Forkmann, 1991).

The biosynthetic pathway for the flavonoid pigments (hereinafter referred to as the "flavonoid pathway") is well established (Ebel and Hahlbrock, 1988; Hahlbrock and Grisebach, 1979; 20 Wiering and de Vlaming, 1984; Schram *et al.*, 1984; Stafford, 1990). The first committed step in the pathway involves the condensation of three molecules of malonyl-CoA with one molecule of *p*-coumaroyl-CoA. This reaction is catalysed by the enzyme chalcone synthase (CHS). The product of this reaction, 2',4,4',6'-tetrahydroxychalcone, is normally rapidly isomerized to produce naringenin by the enzyme chalcone-flavanone isomerase (CHI). 25 Naringenin is subsequently hydroxylated at the 3-position of the central ring by flavanone 3-hydroxylase (F3H) to produce dihydrokaempferol (DHK).

The B-ring of dihydrokaempferol (DHK) can be hydroxylated at either the 3', or both the 3' and 5' positions, to produce dihydroquercetin (DHQ) and dihydromyricetin (DHM), 30 respectively (see Figure 1). DHQ is an intermediate required for the production of cyanidin-

based anthocyanins and DHM is an intermediate required for the production of delphinidin-based anthocyanins in the flavonoid pathway. Two key enzymes involved in this pathway are flavonoid 3'-hydroxylase (F3'H) and flavonoid 3',5'-hydroxylase (F3'5'H). The F3'H acts on DHK to produce DHQ. The F3'5'H is a broad spectrum enzyme catalyzing hydroxylation of DHK in the 3' and 5' positions and of DHQ in the 5' position (Stotz and Forkmann, 1982), in both instances producing DHM. The pattern of hydroxylation of the B-ring of anthocyanins plays a key role in determining petal colour.

Another key enzyme is dihydroflavonol-4-reductase (DFR) which has variable substrate specificity depending on its plant source and has the potential to act on any one or more of DHK, DHQ and DHM.

Many of the major cutflower species lack the F3'5'H and consequently cannot display the range of colours, resultant from synthesis of delphinidins and derivatives thereof, that would otherwise be possible. This is particularly the case for carnations which constitute a major proportion of the world-wide cutflower market. There is a need, therefore, to modify carnation plants to generate transgenic plants which are capable of producing the F3'5'H, thereby providing a means of converting DHK and DHQ to DHM, thereby influencing the hydroxylation pattern of the anthocyanins and allowing the production of anthocyanins derived from delphinidin. Flower colour is modified as a result and a single species is able to express a broader spectrum of flower colours.

In work leading up to the present invention, the inventors sought to genetically manipulate the flavonoid pathway in carnation plants to generate a range of plants with the capacity to direct DHK metabolism towards delphinidin in preference to or rather than pelargonidin or cyanidin. The resulting plants exhibit altered flower colouration relative to presently available carnation plants. The new transgenic carnation plants and more particularly flowers cut therefrom fulfil a long-felt need in the horticultural and more particularly the floricultural industry in relation to carnations. The technology of the present invention is also applicable to a range of other flowering plants such as roses and chrysanthemums.

- 4 -

Accordingly, one aspect of the present invention contemplates a method for producing a plant exhibiting altered flower colour, said method comprising selecting a plant which is substantially incapable of synthesizing a DFR which acts on DHK and introducing into said selected plant one or more genetic constructs comprising nucleotide sequences encoding a 5 F3'5'H and a DFR which is capable of acting on DHM but substantially incapable of acting on DHK.

Accordingly, one aspect of the present invention contemplates a method for producing a plant exhibiting altered flower colour, said method comprising selecting a plant from which said 10 first plant is to be derived wherein said selected plant is substantially incapable of synthesizing a DFR which acts on DHK and introducing into said selected plant one or more genetic constructs comprising nucleotide sequences encoding a F3'5'H and a DFR which is capable of acting on DHM but substantially incapable of acting on DHK.

15 More particularly, the present invention provides a method for producing a carnation plant exhibiting altered flower colour, said method comprising selecting a carnation plant from which said first carnation plant is to be derived wherein said selected plant is substantially incapable of synthesizing a DFR which acts on DHK in flowers and introducing into said selected plant one or more genetic constructs comprising nucleotide sequences encoding a 20 F3'5'H and a DFR which is capable of acting on DHM but substantially incapable of acting on DHK.

The present invention is exemplified herein using carnation plants. This is done, however, with the understanding that the instant invention extends to a range of flowering plants such 25 as but not limited to roses and chrysanthemums. Reference hereinafter to carnations should be taken as reference to other suitable flowering plants.

Another aspect of the present invention is directed to a method for producing a carnation plant exhibiting altered flower colour, said method comprising the steps of:

- 5 -

- (i) selecting a plant from which said carnation plant is to be derived wherein said selected plant is substantially incapable of synthesizing a DFR which acts on DHK in flowers;
- 5 (ii) transforming cells of said selected plant with one or more genetic constructs comprising nucleotide sequences which encode separately F3'5'H and DFR provided said DFR is capable of acting on DHM but substantially incapable of acting on DHK;
- 10 (iii) regenerating a transgenic plant from said transformed cells such that said regenerated plant is capable of expressing said F3'5'H and said DFR of step (ii); and
- (iv) growing said plant under conditions to permit expression of said F3'5'H and said DFR of step (ii) in flowers.

Reference herein to altered flower colour includes alteration in the colour of any or all 15 components of the flower including the petal, sepal and stamen. Conveniently, altered flower colour is shown by comparing the flower colour of a transgenic plant made in accordance with the present invention with a plant of the same species but which possesses a DFR which can act on DHK. In practical terms, a comparison may be made between the transgenic plant and the "selected" plant, i.e. the plant from which the transgenic plant is derived.

20 The present invention is predicated in part on the genetic manipulation of the anthocyanin pathway in carnation plants to direct metabolism of DHK preferentially towards DHM and delphinidin and derivatives thereof rather than through leucopelargonidin and pelargonidin derivatives or DHQ and leucocyanidin and cyanidin derivatives. Flowers of carnation plants 25 lack a F3'5'H and, hence, DHK is unable to undergo metabolism down the delphinidin pathway which is required in order to produce flower colours in the range covering lilac, violet, purple and blue or various shades thereof. To achieve a preferential re-direction of DHK metabolic products down the delphinidin pathway, the inventors screened for and located white lines of carnations lacking a functional DFR. Expression of an introduced 30 nucleic acid molecule results in a F3'5'H which is capable of directing DHK metabolism to

- 6 -

DHM. Introduction and expression of a nucleic acid molecule encoding a non-indigenous DFR which is capable of acting on DHM but not DHK, then results in DHM metabolism being directed to leucodelphinidin thereby allowing subsequent conversion to other derivatives of delphinidin. Little or no metabolism occurs *via* the pelargonidin or cyanidin pathway.

5

In a particularly preferred embodiment, the flowers of the starting plant also lack a F3'H.

According to this preferred embodiment, the present invention contemplates a method for producing a carnation plant exhibiting altered flower colour properties, said method 10 comprising selecting a carnation plant from which said first mentioned carnation plant is to be derived wherein said selected plant is substantially incapable of synthesizing either a F3'H or a DFR which can act on DHK in flowers and introducing into said selected plant one or more genetic constructs comprising nucleotide sequences encoding an F3'5'H and a DFR which is capable of acting on DHM but substantially incapable of acting on DHK.

15

More particularly, this aspect of the present invention is directed to a method for producing a carnation plant exhibiting altered flower colour, said method comprising the steps of:

- (i) selecting a carnation plant from which said first mentioned carnation plant is to be derived, wherein said selected plant is substantially incapable of synthesizing either F3'H or a DFR which can act on DHK in flowers;
- 20 (ii) transforming cells of said selected plant with one or more genetic constructs comprising nucleotide sequences which encode separately F3'5'H and DFR provided said DFR is capable of acting on DHM but substantially incapable of acting on DHK;
- 25 (iii) regenerating a transgenic plant from said transformed cells such that said regenerated plant is capable of expressing said F3'5'H and said DFR of step (ii); and
- (iv) growing said plant under conditions to permit expression of said F3'5'H and said DFR of step (i) in flowers.

30

The present invention is exemplified herein using petunia DFR as an enzyme capable of acting on DHM but not DHK. This is done, however, with the understanding that the present invention extends to a DFR enzyme from any plant providing it is capable of acting on DHM but not DHK. Similarly, a particularly useful F3'5'H is from petunia but other sources of 5 F3'5'H include egg plant, lisianthus, gentian, pansy, china aster, anemone, grape, iris, hyacinth, delphinium and bell flower.

Reference herein to the ability of a plant to produce or not produce an enzyme such as DFR, F3'H and F3'5'H relates to its ability in flowers and not necessarily elsewhere in the plant.

10

A particularly preferred embodiment of the present invention relates to a method for producing a carnation plant exhibiting altered flower colour, said method comprising selecting a carnation plant from which said first carnation plant is to be derived wherein said selected plant is substantially incapable of synthesizing either F3'H or a DFR which can act on DHK 15 in flowers, introducing into cells of said selected plant one or more genetic constructs comprising nucleotide sequences which separately encode a F3'5'H and a petunia DFR, regenerating a plant from said cells, and then growing said plant under conditions sufficient to permit expression of said F3'5'H and said petunia DFR such that said plant produces flowers of different colour relative to said selected plant. Preferably, the F3'5'H is of petunia 20 origin.

The selected plant from which the subject transgenic plant exhibiting altered flower colour is derived may be a natural mutant for DFR such that it substantially does not produce DFR or produces reduced levels of this enzyme, or produces the enzyme with altered substrate 25 specificities such as substantial inability to metabolize DHK. Preferably, however, the selected plant is a double mutant for DFR and F3'H. Mutants of these types would generally have white flowers. Mutations may be single or multiple nucleotide substitutions, deletions and/or additions to the nucleotide sequences defining the enzymes. Alternatively, the mutations may be induced or directed by, for example, transposon tagging, oligonucleotide- 30 directed mutagenesis, *Agrobacterium*-directed mutagenesis or viral-directed mutagenesis.

- 8 -

Alternatively, genetic constructs may be introduced to reduce expression of DFR and/or F3'H by, for example, antisense or co-suppression methods. In this aspect of the invention, a plant may be chosen with substantial inability to synthesize one of DFR or F3'H and a genetic sequence introduced to reduce expression of the other of said DFR or F3'H. Alternatively,

5 a plant may be selected with substantial inability to synthesise one of DFR or F3'H and a mutation induced in the other of said DFR or F3'H, by any of the means mentioned above. In either case, the resulting plant would be a "selected" plant and a recipient for a F3'5'H and a DFR capable of acting on DHM but not DHK. A "selected" plant may also be considered a "parent" plant since it is from this plant that a transgenic plant exhibiting altered flower

10 colour is derived in accordance with the methods of the present invention.

The altered flower colour contemplated by the present invention includes the ability of the carnations to produce a range of colours including lilac, violet, purple and blue flowers or various shades thereof from, for example, deep mauve to dark blue to a violet colour or their
15 various shades or combinations thereof.

Another aspect of the present invention contemplates a transgenic plant exhibiting altered flower colour, said plant being substantially incapable of synthesizing a DFR which acts on DHK and which carries a nucleic acid molecule comprising a sequence of nucleotides which
20 encodes a F3'5'H and a DFR which is capable of acting on DHM but substantially incapable of acting on DHK.

Preferably, the plant is a carnation, rose, gerbera or chrysanthemum. In accordance with this aspect of the present invention, the nucleic acid molecule may comprise multiple genetic
25 constructs separately encoding DFR and F3'5'H or a single combined genetic construct encoding both enzymes but with expression being generally directed by two separate promoters. The term "nucleic acid molecule" encompasses single or multiple nucleic acid molecules.

- 9 -

In a preferred aspect of the present invention, there is provided a carnation plant exhibiting the following properties:

- (i) a substantial inability to synthesize a DFR which acts on DHK;
- (ii) an ability to synthesize a non-indigenous DFR which is capable of acting on DHM but is substantially incapable of acting on DHK; and
- (iii) an altered flower colour relative to a carnation plant which expresses a DFR which acts on DHK.

Preferably, the carnation plant is also substantially incapable of synthesizing a F3'H.

10 According to this preferred embodiment, there is provided a carnation plant exhibiting the following properties:

- (i) a substantial inability to synthesize a F3'H;
- (ii) a substantial inability to synthesize a DFR which acts on DHK;
- (iii) an ability to synthesize a non-indigenous DFR which is capable of acting on DHM but is substantially incapable of acting on DHK; and
- (iv) an altered flower colour relative to a carnation plant which expresses a F3'H and/or a DFR which acts on DHK.

The present invention extends to the flowers and in particular flowers cut from such 20 transgenic carnations or from plants produced according to the methods herein disclosed.

A "non-indigenous" DFR is an enzyme not normally produced in carnation plants and, in one preferred embodiment, is from petunia. A "non-indigenous" DFR may alternatively have originated from a carnation plant but has undergone mutation to restrict its substrate 25 specificity to DHM. The F3'S'H is also non-indigenous and in one aspect is preferably from petunia.

Another aspect of the present invention contemplates a method for producing carnation flowers exhibiting altered flower colour, said method comprising growing a transgenic 30 flowering carnation plant for a time and under conditions for flowers to form and then

optionally harvesting said flowers, said transgenic carnation plant having been genetically manipulated such that:

5 (i) it is substantially incapable of expressing a DFR which is capable of acting on DHK;

(ii) it is capable of expressing a non-indigenous F3'5'H; and

(iii) it is capable of expressing a non-indigenous DFR which is capable of acting on DHM but is substantially incapable of acting on DHK.

In accordance with this aspect of the present invention, the flowers exhibiting altered flower colour will now have the capacity to metabolise DHK to DHM and delphinidin and derivatives thereof of the flavonoid pathway.

Preferably, the transgenic plant is also substantially incapable of expressing a F3'H.

15 Other aspects of the present invention include the use of genetic sequences encoding separately a F3'5'H, a DFR which is capable of acting on DHM but is substantially incapable of acting on DHK, and optionally also a F3'H, in the genetic manipulation of a carnation plant substantially incapable of expressing or synthesizing an active DFR capable of acting on DHK or DHM, so as to allow the manufacture of a carnation plant exhibiting altered 20 flower colour compared to a carnation plant which is capable of synthesizing a DFR capable of acting on DHK. In a related aspect, the present invention extends to flowers and in particular flowers cut from said carnation plants.

The present invention is further described by reference to the following figures and/or examples.

In the Figures:

- 11 -

Figure 1 is a schematic diagram of the pathways for conversion of dihydroflavonols to flavonols and anthocyanins. Abbreviations: DHK = dihydrokaempferol, DHQ = dihydroquercetin, DHM = dihydromyricetin, K = kaempferol, Q = quercetin, M = myricetin, F3'H = flavonoid 3'-hydroxylase, F3'5'H = flavonoid 3',5'-hydroxylase, FLS 5 = flavonol synthase, DFR = dihydroflavonol-4-reductase, ANS = anthocyanidin synthase, 3GT = flavonoid 3-glucosyltransferase.

Figure 2 is an autoradiographic representation of a Northern analysis comparing the expression levels of DFR and ANS mRNA in thirteen commercially-available white carnation 10 cultivars. Arrows point to the relevant transcripts.

Figure 3 is a diagrammatic representation of the binary expression vector pCGP1470, construction of which is described in Example 9. Tc resistance = the tetracycline resistance gene; LB = left border; RB = right border; surB = the coding region and terminator 15 sequences for the tobacco acetolactate synthase gene; 35S = the promoter region from the cauliflower mosaic virus 35S gene; CHS = the promoter region from the snapdragon chalcone synthase gene; Hf1 = the DNA sequence encoding petunia flavonoid 3',5'-hydroxylase; D8 = terminator sequence from a petunia phospholipid transferase; MAC = the mannopine synthase promoter enhanced with cauliflower mosaic virus 35S gene 20 sequences; DFR = the DNA sequence encoding dihydroflavonol-4-reductase; mas = the terminator sequence from the *Agrobacterium tumefaciens* mannopine synthase gene. Selected restriction enzyme sites are indicated.

Figure 4 is a diagrammatic representation of the binary expression vector pCGP1473, 25 construction of which is described in Example 10. Tc resistance = the tetracycline resistance gene; LB = left border; RB = right border; surB = the coding region and terminator sequences for the tobacco acetolactate synthase gene; 35S = the promoter region from the cauliflower mosaic virus 35S gene; CHS = the promoter region from the snapdragon chalcone synthase gene; Hf1 = the DNA sequence encoding petunia flavonoid 3',5'-hydroxylase; D8 = terminator sequence from a petunia phospholipid transferase; genomic 30

DFR = the genomic DNA sequence encoding dihydroflavonol-4-reductase. Selected restriction enzyme sites are indicated.

Figure 5 is an autoradiographic representation of a Southern hybridization of DNA isolated 5 from leaf tissue from White Unesco, which had been transformed with a genetic construct (pCGP1470) containing the tobacco acetolactate synthase gene as selectable marker, and the nucleic acid molecule encoding F3'5'H and DFR. Carnation genomic DNA was digested with the restriction endonuclease *Xba*I and the Southern blot was probed with a ³²P-labelled 730 base-pair fragment of the petunia F3'5'H coding region. Filters were washed in 0.2 x 10 SSC/1% w/v SDS at 65°C. Lanes 1-12 represent DNA samples isolated from independent transgenic plants whilst lane 13 is non-transformed White Unesco (negative control). No bands were detected in the non-transformed negative control. Lane 14 represents 33 pg of pCGP1470 plasmid DNA digested with *Xba*I.

15 Figure 6 is an autoradiographic representation of a Northern blot of F3'5'H and DFR RNA in petals. Total RNA (10 μ g/lane) was analysed from petals of White Unesco plants transformed with pCGP1470 (lanes 1-7), and petals of non-transgenic White Unesco flowers (lane 8). No bands were detected in the non-transformed negative control in lane 8. Lane 9 represents 10 μ g of RNA isolated from *Petunia hybrida* cv. Old Glory Blue. A: Northern 20 blot was hybridized with ³²P-labelled DNA from a 730 bp *Eco*RV fragment of a petunia F3'5'H cDNA clone and washed in 2 x SSC/1% w/v SDS at 65°C for 0.5 hour. B: Northern blot was hybridized with a ³²P-labelled 1.2 kb *Sac*I/*Xba*I DNA fragment of a petunia DFR cDNA clone from plasmid pCGP 1403 (see Example 8a).

25 Figure 7 is a black and white representation of a colour photographic plate representing a non-transgenic control White Unesco flower (the white flower on the left) and a flower from a White Unesco plant transformed with pCGP1470. The transformed plant produces a flower which is lilac/violet in colour.

30 Original colour plates are available for inspection from the Applicant.

EXAMPLE 1

Strategy for altering flower colour using DFR mutant cultivars

The flowers of some plants such as, for example, rose, carnation and gerbera, produce two types of anthocyanidins, depending on their genotype - pelargonidin and cyanidin. In the absence of F3'H activity, pelargonidin is produced; in its presence, cyanidin is produced. Pelargonidin is usually accompanied by kaempferol, a colourless flavonol. Cyanidin pigments are usually accompanied by either quercetin or both kaempferol and quercetin. Both pelargonidin and kaempferol are derived from DHK; both cyanidin and quercetin are derived from DHQ (Figure 1). A number of enzymes, including DFR, ANS and 3GT are required for the conversion of dihydroflavonols (DHK, DHQ and DHM) to the coloured anthocyanins. A third type of anthocyanidin, delphinidin, cannot be produced in flowers of these plants, owing to the natural absence of F3'S'H activity.

In carnation, the DFR enzyme is capable of metabolising two dihydroflavonols, DHK and DHQ, to leucoanthocyanidins which are ultimately converted to anthocyanidin pigments which are responsible for flower colour. DHK is converted to leucopelargonidin giving rise to red-coloured carnations and DHQ to leucocyanidin producing crimson carnations (Geissman and Mehlquist, 1947; Stich *et al.*, 1992b) (see Figure 1). Carnation DFR is also capable of converting DHM to leucodelphinidin (Forkmann *et al.*, 1987). However, naturally-occurring carnation lines do not contain a flavonoid 3',5'-hydroxylase enzyme and therefore do not synthesise DHM.

The petunia DFR enzyme has a different specificity to that of the carnation DFR. It is able to convert DHQ through to leucocyanidin, but it is not able to convert DHK to leucopelargonidin (Forkmann *et al.*, 1987). In petunia lines containing the F3'S'H enzyme, the petunia DFR enzyme can convert the DHM produced by the action of F3'S'H to leucodelphinidin, which is further modified giving rise to delphinidin - the pigment responsible for blue-coloured flowers (see Figure 1). Even though the petunia DFR is capable of converting both DHQ and DHM, it is able to convert DHM far more efficiently, favouring

the production of delphinidin (Forkmann *et al.*, 1987).

Carnation is transformable with a nucleic acid molecule encoding the petunia F3'5'H from another species, thereby allowing the production of DHM and ultimately the accumulation 5 of delphinidin (International Patent Application No. PCT/AU94/00265; [WO94/28140]). However, the efficiency of delphinidin production in many of these plants is low due to the competition of carnation DFR with the F3'5'H enzyme for DHK or DHQ as substrate. The accumulation of significant amounts of delphinidin may, therefore, be limited by the action of the carnation DFR.

10

The inventors have shown that the efficiency of delphinidin production is markedly increased by transformation of a DFR mutant cultivar with an appropriate genetic construct. In such a line there is no accumulation of anthocyanin, due to the absence of DFR enzyme activity. However, when a nucleic acid molecule encoding the petunia DFR enzyme is used to 15 transform this line, together with a nucleic acid molecule encoding flavonoid 3',5'-hydroxylase and both enzymes are expressed, DHK is then converted to leucodelphinidin by the introduced enzymes and ultimately to delphinidin pigments by the plant's endogenous enzymes. If the DFR mutant line is also a F3'H mutant, there is little or no production of DHQ and therefore the only anthocyanins produced by the transgenic plants are delphinidin 20 derivatives. In the presence of the F3'H, cyanidin pigments may also be produced, but delphinidin is the major anthocyanin produced because the petunia DFR is more efficient at utilizing DHM than DHQ as a substrate. Flowers from carnation plants genetically manipulated in this manner produce a range of flower colours covering lilac, violet, purple and blue or various shades thereof. Exemplification of the application of this strategy in 25 accordance with the present invention in the production of carnations with altered flower colour is provided by the following examples.

- 15 -

EXAMPLE 2

Screening of *Dianthus caryophyllus* (carnation) cultivars

a. Flavonol analysis of carnation flowers

To identify carnation cultivars having genotypes suitable for application of the strategy of 5 Example 1, white-flowered cultivars were obtained. Flavonols were extracted from each and analysed by thin layer chromatography (TLC), as described in Example 16a. The only flavonol detected in all of these cultivars was kaempferol, indicating that F3'H activity was absent, or substantially reduced, in the flowers of all cultivars.

10 b. Northern analysis of white carnation cultivars

Northern analysis was then performed, as described in Example 14. After the RNA was transferred from the gel to a Hybond-N filter (Amersham), the filter was probed with the 290 bp *Bam*HI/*Hind*III cDNA fragment of ANS (described in Example 6) labelled with ^{32}P . The 1.2 kbp *Asp*718/*Bam*HI partial DFR clone (described in Example 7) was also labelled with 15 ^{32}P and used to probe duplicate filters. Prehybridisation (1 hour at 42°C) and hybridization (16 hours at 42°C) were carried out as described in Example 14. Filters were washed in 2 x SSC, 1% w/v SDS at 65°C for 1 to 2 hours and then 0.2 x SSC, 1% w/v SDS at 65°C for 0.5 to 1 hour. The Northerns were autoradiographed overnight at -70°C.

20 This analysis indicated that two of the tested carnation cultivars had no DFR message, while still producing significant levels of ANS mRNA (see Figure 2). These two carnation cultivars, White Unesco and White Diana, thus appeared to be of the correct genotype for application of this strategy.

25 c. Leucoanthocyanidin feeding experiments

To confirm that White Diana and White Unesco were not mutated in any of the genes necessary for conversion of leucoanthocyanidin to anthocyanin, precursor feeding experiments were carried out. Petal segments of White Unesco and White Diana were placed in 1 mg/mL solutions of leucopelargonidin and leucocyanidin and incubated for 16 hours at room 30 temperature. Anthocyanin synthesis occurred near the cut edges of the petals in each case.

- 16 -

Leucopelargonidin feeding led to the synthesis of pelargonidin, and leucocyanidin led to the synthesis of cyanidin. Anthocyanidin identity was determined by TLC analysis (see Example 16b). Either of these two cultivars was thereby shown to be a suitable candidate for genetic manipulation utilizing the DFR mutant strategy. Further exemplification is provided with cv.

5 White Unesco.

EXAMPLE 3

Biological Reagents

All restriction enzymes and other reagents were obtained from commercial sources and used 10 generally according to the manufacturer's recommendations.

The cloning vector **pBluescript II (KS⁺)** was obtained from Stratagene.

The *Sma*I-cut **pUC18** cloning vector was obtained from Pharmacia.

15

EXAMPLE 4

Bacterial Strains

The bacterial strains used in the following examples were:

Escherichia coli :

20 **XL1-Blue**

supE44, hsdR17(r_k-, m_k +), recA1, endA1, gyrA96(Nal^r), thi-1, relA1, lac-, [F'proAB, lacI_S, lacZΔM15, Tn10(tet^r)] (Bullock *et al.*, 1987).

DH5 α

25 **supE44, Δ(lacZYA-ArgF), U169, Φ80lacZΔM15, hsdR17(r_k-, m_k +), recA1, endA1, gyrA96(Nal^r), thi-1, relA1, deoR** (Hanahan, 1983; BRL, 1986).

Agrobacterium tumefaciens:

AGL0 Lazo *et al.* (1991)

- 17 -

EXAMPLE 5

Plant Growth Conditions

Unless otherwise stated, plants were grown in specialised growth rooms with a 14 hour day length at a light intensity of 10,000 lux minimum and a temperature of 22°C to 26°C.

5

EXAMPLE 6

Isolation of a carnation anthocyanidin synthase cDNA clone

a. Polymerase Chain Reaction primers

A Polymerase Chain Reaction (PCR) method was employed to isolate a DNA fragment 10 representing carnation anthocyanidin synthase (ANS). Degenerate oligonucleotides were designed to conserved regions of 2-oxoglutarate-dependent dioxygenase sequences from plants. Oligonucleotides were synthesized on an Applied Biosystems PCR-Mate DNA synthesiser using phosphoramidite chemistry. The oligonucleotides synthesized were:

15 5' AC(A,G)TC(A,G)GT(A,G)TGIGC(T,C)TCIACICC 3' SEQ ID NO:1
5' TGGGA(A,G)GA(T,C)TA(T,C)ITITT(T,C)CA 3' SEQ ID NO:2

b. Isolation of an ANS fragment from carnation petals

Total RNA was extracted from petals of carnation cv. Laguna flowers at stage 1 (Stich *et al.*, 20 1992a) using the method of Turpen and Griffith (1986). Oligo dT (12-18)-primed cDNA was synthesized from 50 µg of total RNA by Superscript™ (BRL) according to the manufacturer's instructions. The cDNA was purified by S200 spun-column chromatography (Pharmacia) followed by ethanol precipitation. PCR amplification was carried out on 50 ng of cDNA in the presence of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 0.01% gelatine, 0.2 25 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 0.4 µM each primer and 1.25 units *Taq* polymerase (Cetus). The reaction mix (50 µL) was cycled once at each of 95°C for 3 min; 48°C for 1 min; 72°C for 1 min; and then 39 times at each of 95°C for 1 min; 48°C for 1 min; 72°C for 1 min. A DNA fragment of 290 base pairs (bp) was produced.

- 18 -

c. Sequence analysis of carnation ANS fragment

The 290 bp fragment was isolated by Sea Plaque™ low gelling temperature agarose (FMC) electrophoresis in a TAE running buffer (40 mM Tris, 50 mM acetic acid, 50 mM EDTA). The DNA was extracted from the agarose by heating to 65°C, phenol extraction and ethanol 5 precipitation. The fragment was then ligated into a ddT-tailed vector prepared as described by Holton and Graham (1991). Sequencing of plasmid clones was performed using Prism™ Ready Reaction Dye Primer chemistry and a DNA Sequencing System 373A (Applied Biosystems). When compared with the petunia ANS cDNA (Weiss *et al.*, 1993) using FASTA (Pearson and Lipman, 1988) the carnation sequence showed 83% homology at the 10 amino acid level. This 290 bp cDNA fragment was used in Northern analysis of a range of white carnation cultivars (as described in Example 2b).

EXAMPLE 7

Isolation of a carnation dihydroflavonol-4-reductase (DFR) cDNA clone

15 a. Construction of a cDNA library

Total RNA was extracted from carnation cv. Laguna petals at stage 9 (Stich *et al.*, 1992a). Polyadenylated RNA was selected using the Oligotex (Qiagen) purification system according to the manufacturer's instructions. A directional cDNA library was constructed using 2 µg of poly(A)⁺ RNA, as a template for cDNA synthesis, and Superscript™ (BRL) according to 20 the manufacturer's instructions. DNA Polymerase I (Klenow fragment) was used to synthesize second strand cDNA which was blunted and ligated to *Eco*RI adapters. After digestion with *Xba*I, the cDNA was size-fractionated on a S200 column (Pharmacia). One third of the cDNA was ligated with 1 µg of Uni-Zap™ XR vector (Stratagene). Ligation was carried out at 4°C for 4 days and then packaged using Packagene™ (Promega). The resultant library contained 25 1.5 x 10⁵ plaque forming units (p.f.u.) and was amplified by eluting the bacteriophage from the agar plates into phage storage buffer (100 mM NaCl, 10 mM MgCl₂, 10 mM Tris-HCl (pH 7.4), 0.05% w/v gelatine).

b. Screening of the cDNA library

Approximately 100,000 p.f.u. were plated (at 10,000 pfu/plate) onto NZY plates and incubated at 37°C for 8 hours and then at 4°C for 1 hour. Thereafter, duplicate colony lifts were taken onto Colony/Plaque Screen™ filters (DuPont) and treated as recommended by the manufacturer. Prior to hybridization, the duplicate filters were prewashed in a solution of 50 mM Tris-HCl pH 8.0, 1 M NaCl, 1 mM EDTA, 0.1% w/v sarcosine (prewashing solution) at 42°C for 30 minutes followed by similar washes in 0.4 M NaOH and in neutralising solution (0.5 M Tris-HCl pH 8.0, 1.5 M NaCl). After rinsing in 2 x SSC, the colony lifts were prehybridized (42°C, 1 hour) and hybridized (42°C, overnight) in a solution of 6 x SSC (0.6 M NaCl, 0.06 M sodium citrate), 0.5% w/v sodium dodecyl sulphate (SDS), 0.1% polyvinyl-pyrrolidone (PVP), 0.1% w/v bovine serum albumin (BSA), 0.1% ficoll, 0.01 M ethylenediaminetetra-acetic acid (EDTA), and 100 mg/mL denatured herring sperm DNA. The full-length ³²P-labelled petunia dihydroflavonol 4-reductase (DFR) cDNA clone from pCGP1403 (Example 8) was used for hybridization. Filters were washed at 65°C in 2 x SSC/1% w/v SDS and exposed to Kodak XAR film with an intensifying screen at -70°C for 16 hours.

c. Sequence analysis of the carnation DFR cDNA clone

The clones isolated were sequenced using Prism™ Ready Reaction Dye Primer chemistry and 20 a DNA Sequencing System 373A (Applied Biosystems). One clone was found to have homology with the petunia DFR. This clone contained a 1.2 kilobase pair (kbp) cDNA insert and appeared to be a partial DFR clone only.

Double-stranded DNA sequence of the entire cDNA insert was obtained using a shotgun clone 25 sequencing strategy. The DFR cDNA fragment was purified, self-ligated and sheared by ultrasound (four times seven-second bursts at 20 watts from a Branson sonicator with a microprobe attached). The fragment ends were repaired using T4 DNA polymerase and size fractionated in the range of 350 to 600 bp using agarose gel electrophoresis in TAE running buffer. Fragments were purified using Geneclean (Bio101), ligated into *Sma*I-cut pUC18 and 30 individual clones were sequenced. Comparison of the carnation sequence with the Swissprot

- 20 -

protein database using the FASTA program (Pearson and Lipman, 1988) showed that there was 66.9% identity at the amino acid level with the petunia DFR cDNA and 65.7% with the snapdragon DFR. This 1.2 kbp partial DFR clone was used in Northern analysis of a range of white carnation cultivars (as described in Example 2b).

5

EXAMPLE 8

Isolation of petunia DFR genetic sequences

a. Isolation of a functional DFR cDNA clone

To isolate a full-length petunia DFR cDNA, 200,000 clones of a *Petunia hybrida* cv. Old 10 Glory Blue λZAP cDNA library (Holton *et al.*, 1993) were screened using a ³²P-labelled 1 kb fragment of a petunia DFR cDNA clone described previously (Brugliera *et al.*, 1994). Twenty clones hybridized strongly with this probe. These clones were picked and plasmids were excised *in vitro*. DNA sequence analysis revealed that eight of these clones contained the entire protein coding region of DFR, when compared with published sequences (Beld *et* 15 *al.*, 1989; Huits *et al.*, 1994).

One of the eight full-length petunia DFR cDNA clones, contained in plasmid pCGP1403, was used to screen a carnation cDNA library, as described in Example 7. This full-length clone was further subcloned into a plant expression vector to test for function, as follows. A 1.5 20 kb *Asp*718/*Bam*HI fragment of pCGP1403, which contained the DFR cDNA, was ligated with an *Asp*718/*Bam*HI digest of the vector pCGP40 (International Patent Application No. PCT/AU92/00334 [WO 93/01290]. The resulting plasmid (pCGP1406) contained the petunia DFR cDNA between the MAC promoter (Comai *et al.*, 1990) and the mannopine synthase (mas) terminator (Comai *et al.*, 1990). A *Bgl*II digest of this plasmid was used in the 25 construction of pCGP1470, as described in Example 9.

The cDNA clone in plasmid pCGP1406 was shown to be functional by bombardment of *Petunia hybrida* cv. Br140 petals. Br140 lacks DFR activity due to a mutation at the *an6* genetic locus, so the flowers are white and do not produce anthocyanins. However, after 30 bombardment of petals with pCGP1406, coloured cells were produced due to anthocyanin

synthesis, indicating that the DFR gene fragment present in this plasmid was functional.

b. Isolation of a functional DFR genomic clone

A genomic library was made from *Petunia hybrida* cv. Old Glory Blue DNA in the vector 5 λ 2001 (Holton, 1992). Approximately 200,000 p.f.u. were plated out on NZY plates, lifts were taken onto NEN filters and the filters were hybridised with 400,000 cpm/mL of the 32 P-labelled 1kb petunia DFR cDNA fragment (see 8a, above). Hybridising clones were purified, DNA was isolated from each and mapped by restriction enzyme digestion. A 13 kb *SacI* fragment of one of these clones was isolated and ligated with *SacI*-cut pBluescriptII to create 10 pCGP1472. The genomic clone in plasmid pCGP1472 was also shown to be functional by bombardment of *Petunia hybrida* cv. Br140 petals. After bombardment of petals with pCGP1472, coloured cells were produced, due to anthocyanin synthesis. Finer mapping indicated that a 5 kb *BglII* fragment contained the entire DFR gene. This 5kb fragment was used in the construction of pCGP1473, as described in Example 10.

15

EXAMPLE 9

Construction of pCGP1470

Plasmid pCGP485 (International Patent Application PCT/AU94/00265 [WO94/28140] was digested with *PstI* to release a 3.5 kb genetic construct consisting of a snapdragon CHS 20 promoter sequence, a petunia *Hf1* cDNA fragment and a petunia phospholipid transfer protein terminator sequence (International Patent Application PCT/AU92/00334 [WO93/01290]). The overhanging 3'-ends of the fragment were removed with T4 DNA polymerase according to standard protocols (Sambrook *et al.*, 1989) before ligation into the *SmaI* site of the binary vector pWTT2132 (DNAP). The resultant clone was designated 25 pCGP1452.

A 3.4 kb genetic construct containing the MAC promoter (Comai *et al.*, 1990), a petunia DFR cDNA (Example 15) and the mannopine synthase (mas) terminator (Comai *et al.*, 1990), was isolated from pCGP1406 as a *BglII* fragment (see Example 8a). The resulting 5'- 30 overhang was "filled in" using DNA Polymerase I (Klenow fragment) according to standard

- 22 -

protocols (Sambrook *et al.*, 1989). The fragment was then ligated into *Pst*I restricted, T4 DNA polymerase-treated pCGP1452 to create pCGP1470. A map of pCGP1470 is presented in Figure 3.

5

EXAMPLE 10

Construction of pCGP1473

A 5 kb *Bgl* II fragment from a petunia DFR genomic clone, pCGP1472 (described in Example 8b), was isolated and the resulting 5'-overhang was "filled-in" using DNA Polymerase I (Klenow fragment). The fragment was ligated into the *Pst*I-restricted, T4 DNA 10 polymerase-treated pCGP1452 to create pCGP1473. A map of pCGP1473 is presented in Figure 4.

EXAMPLE 11

Transformation of *E. coli* and *A. tumefaciens*

15 *Escherichia coli* strains DH5 α and XL1-Blue, used for routine manipulations, were transformed using the method of Inoue *et al.* (1990).

The plasmids pCGP1470 and pCGP1473 were introduced into *Agrobacterium tumefaciens* strain AGL0 by adding 5 μ g of plasmid DNA to 100 μ L of competent *Agrobacterium* 20 *tumefaciens* cells prepared by inoculating a 50 mL MG/L (Garfinkel and Nester, 1980) culture and growing for 16 hours with shaking at 28°C. The cells were then pelleted and resuspended in 1 mL of 20 mM CaCl₂. The DNA-*Agrobacterium* mixture was frozen by incubation in liquid nitrogen for 2 min and then allowed to thaw by incubation at 37°C for 5 min. The cells were then mixed with 1 mL of MG/L media and incubated with shaking for 25 4 hours at 28°C. Cells of *A. tumefaciens* carrying pCGP1470 or pCGP1473 were selected on MG/L agar plates containing 50 μ g/mL tetracycline. The presence of the plasmid was confirmed by Southern analysis of DNA isolated from the tetracycline-resistant transformants.

EXAMPLE 12

Transformation of *Dianthus caryophyllus* (carnation) cv. White Unesco with nucleic acid molecules encoding DFR and F3'5'H

a. Plant Material

5 Carnation cv. White Unesco cuttings were obtained from Van Wyk and Son Flower Supply, Victoria, Australia. The outer leaves were removed and the cuttings were sterilised briefly in 70% v/v ethanol followed by 1.25% w/v sodium hypochlorite (with Tween 20) for 6 min and rinsed three times with sterile water. All the visible leaves and axillary buds were removed under the dissecting microscope before co-cultivation.

10

b. Co-cultivation of *Agrobacterium* and Carnation Tissue

Agrobacterium tumefaciens strain AGL0 (Lazo *et al.*, 1991), containing the binary vector pCGP1470 or pCGP1473, was maintained at 4°C on LB agar plates with 50 mg/L tetracycline. A single colony was grown overnight in liquid LB broth containing 50 mg/L 15 tetracycline and diluted to 5 x 10⁸ cells/mL next day before inoculation. Carnation stem tissue was co-cultivated with *Agrobacterium* for 5 days on MS medium supplemented with 3% w/v sucrose, 0.5 mg/L benzylaminopurine, 0.5 mg/L 2,4-dichlorophenoxy-acetic acid (2,4-D), 100 µM acetosyringone and 0.25% w/v Gelrite (pH 5.7).

20 c. Recovery of Transgenic Carnation Plants

For selection of transformed stem tissue, the top 6-8 mm of each co-cultivated stem was cut into 3-4 mm segments, which were then transferred to MS medium (Murashige and Skoog, 1962) supplemented with 0.3% w/v sucrose, 0.5 mg/L BAP, 0.5 mg/L 2,4-D, 1 µg/L chlorsulfuron, 500 mg/L ticarcillin and 0.25% w/v Gelrite. After two-three weeks, explants 25 were transferred to fresh MS medium containing 0.3% sucrose, 0.16 mg/L thidiazuron (TDZ), 0.5 mg/L indole-3-butyric acid (IBA), 2 µg/L chlorsulfuron, 500 mg/L ticarcillin and 0.25% w/v Gelrite and care was taken at this stage to remove axillary shoots from stem explants. After 3 weeks, healthy adventitious shoots were transferred to hormone free MS medium containing 3% w/v sucrose, 3 µg/L chlorsulfuron, 500 mg/L ticarcillin, 0.25% w/v 30 Gelrite. Shoots which survived 3 µg/L chlorsulfuron were transferred to MS medium

- 24 -

supplemented with 3% w/v sucrose, 500 mg/L ticarcillin, 5 μ g/L chlorsulfuron and 0.25% w/v Gelrite for shoot elongation.

After 2-3 weeks, leaves were pulled from the shoots which had survived selection and were placed on a regeneration medium consisting of MS medium supplemented with 0.22 mg/L TDZ, 0.5 mg/L IBA, 3 µg/L chlorsulfuron, 500 mg/L ticarcillin and 0.25% w/v Gelrite, to obtain shoot regeneration in the presence of selection. Regenerated shoots were transferred to hormone-free MS medium containing 5 µg/L chlorsulfuron, 500 mg/L ticarcillin and 0.25% w/v Gelrite for 2-4 weeks, then to hormone-free MS medium containing 200 mg/L ticarcillin and 0.4% w/v Gelrite, in glass jars, for normalisation. Suncaps (Sigma) were placed on top of the glass jars to hasten the normalisation of shoots. All cultures were maintained under a 16 hour photoperiod (120 µE/m²/s cool white fluorescent light) at 23°C±2°C. Normalised shoots, approximately 1.5-2.0 cm tall, were rooted on 3 g/kg IBA rooting powder and acclimatised under mist. A soil mix containing 75% perlite/25% peat was used for acclimation, which was carried out at 23°C under a 14 hour photoperiod (200 µE/m²/s mercury halide light) and typically lasted 3-4 weeks. Plants were fertilised with a carnation mix containing 1g/L CaNO₃ and 0.75 g/L of a mixture of microelements plus N:P:K in the ratio 4.7:3.5: 29.2.

20

EXAMPLE 13

Southern Analysis

a. Isolation of Genomic DNA from Carnation

DNA was isolated from 0.3-0.5 grams of leaf tissue using the method of Lassner *et al.* (1989).

25

b. Southern Blots

Approximately 1 μ g of genomic DNA was digested with *Xba*I and electrophoresed through a 1% w/v agarose gel in a running buffer of TAE. The DNA was then denatured in denaturing solution (1.5 M NaCl/0.5 M NaOH) for 0.5 to 1.0 hour, neutralised in 0.5 M Tris-HCl (pH 7.5)/ 1.5 M NaCl for 0.5 to 1.0 hour and the DNA was then transferred to a

- 25 -

Hybond-N (Amersham) filter in 20 x SSC.

Filters were hybridized with ^{32}P -labelled DNA ($10^8 \text{ cpm}/\mu\text{g}$, $2 \times 10^6 \text{ cpm/mL}$) from a 730 bp *EcoRV* fragment of a petunia *Hf1* cDNA clone. Filters were washed in 2 x SSC/1% w/v
5 SDS at 65°C for 1 hour and then 0.2 x SSC/1% w/v SDS at 65°C for 1 hour.

Southern analysis of putative transgenic carnation plants obtained after selection on chlorsulfuron confirmed the integration of the genetic construct comprising the nucleic acid molecule encoding F3'5'H into the genome, as shown in Figure 5. Northern analysis of
10 flowers from carnation plants transformed with pCGP1470, performed as described in Example 14, below, confirmed that the introduced nucleic acid molecules defining F3'5'H and DFR were both expressed (see Figure 6).

EXAMPLE 14

15

Northern Analysis

Total RNA was isolated from tissue that had been frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. An extraction buffer of 4 M guanidine isothiocyanate, 50 mM Tris-HCl (pH 8.0), 20 mM EDTA, 0.1% v/v Sarkosyl, was added to the tissue and the mixture was homogenised for 1 minute using a polytron at maximum speed. After the
20 suspension was filtered through Miracloth, it was centrifuged in a JA20 rotor for 10 minutes at 10,000 rpm. The supernatant was transferred to a clean tube and 0.2 grams of CsCl was added for each mL of supernatant. The CsCl was dissolved by mixing and samples were then layered over a 10 mL cushion of 5.7 M CsCl, 50 mM EDTA (pH 7.0) in 38.5 mL Quick-seal centrifuge tubes (Beckman) and centrifuged at 42,000 rpm for 12-16 hours at 23°C in a Ti-70
25 rotor. Pellets were resuspended in TE/SDS (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% w/v SDS) and extracted with phenol:chloroform (1:1) saturated in 10 mM EDTA (pH 7.5). Following ethanol precipitation the RNA pellets were resuspended in TE/SDS.

RNA samples were electrophoresed through 2.2 M formaldehyde/1.2% w/v agarose gels
30 using running buffer containing 40 mM morpholino-propanesulphonic acid (pH 7.0), 5 mM

- 26 -

sodium acetate, 0.1 mM EDTA (pH 8.0). The RNA was transferred to Hybond-N filters (Amersham) as described by the manufacturer and probed with the appropriate ³²P-labelled cDNA fragment (10⁸ cpm/μg, 2 x 10⁶ cpm/mL). Prehybridization (1 hour at 42°C) and hybridization (16 hours at 42°C) were carried out in 50% v/v formamide, 1 M NaCl, 1% w/v SDS, 10% w/v dextran sulphate. Degraded herring sperm DNA (100 μg/mL) was added with the ³²P-labelled probe for the hybridization step.

5 Filters were washed in 2 x SSC/1% w/v SDS at 65°C for 1 hour and then 0.2 x SSC/1% w/v SDS at 65°C for 1 hour. All filters were exposed to Kodak XAR film with an intensifying 10 screen at -70°C for 48 hours.

EXAMPLE 15

Altered flower phenotype

The expression of the introduced nucleic acid molecules representing F3'5'H and DFR in the 15 DFR/F3'H mutant carnation cultivar White Unesco had a marked effect on flower colour. The flowers of the non-transgenic plants are white, whereas the transgenic plants produced flowers which were a lilac/violet colour. The colour changes observed may also be described in terms of numbers from the Royal Horticultural Society's Colour Chart. In general, the changes can be described as moving the colour from white to the purple/violet/blue hues 20 represented by many, but not all, of the colour squares between 80 and 98.

Although not wishing to limit the possible colour changes which may be achieved, some of the colours observed in flowers of transformed White Unesco plants could be described, approximately, as having changed from white (untransformed) to 84B/C (transformed). 25 Furthermore, the use of a stronger promoter to direct expression of the introduced nucleic acid molecules may allow production of even higher amounts of delphinidin pigment, thereby causing the development of colours of bluer hue. It should be remembered that other biochemical and physiological conditions such as petal pH, extent of co-pigmentation and degree of acylation of anthocyanins will also affect the individual outcome. These can be 30 manipulated by transformation of other suitable cultivars, or by co-transformation with

- 27 -

nucleic acid molecules affecting pH co-pigmentation or acylation. This may enable the development of colours of bluer hue. The citing of specific colours achieved should not be interpreted as defining or limiting the possible range.

5

EXAMPLE 16

Flavonoid analyses

a. TLC analysis of flavonols

Approximately 0.5 gram of fresh carnation petal tissue was added to 1 mL of 2 M HCl in a 1.5 mL microcentrifuge tube (Eppendorf) and heated in a boiling water bath for 30 minutes.

10 Cellular debris was pelleted by centrifugation at 14,000 rpm for 5 minutes and 500 μ L of supernatant was transferred to a clean microcentrifuge tube. Flavonoids were extracted with 200 μ L ethylacetate and centrifuged briefly to separate the phases. The upper (ethylacetate) phase was transferred to a new microcentrifuge tube, dried down under vacuum in a SpeedVac Concentrator (Savant) and resuspended in 15 μ L ethylacetate and a 2 μ L aliquot was 15 spotted onto a cellulose TLC sheet (20 cm x 20 cm, Merck) and run for approximately 3-4 hours in Forestal solvent (30 parts acetic acid: 3 parts HCl: 10 parts water). The TLC plate was then allowed to air dry and the flavonols were viewed under ultraviolet light.

b. TLC analysis of anthocyanidins

20 Two carnation petals were added to 1 mL of 2 M HCl in a 1.5 mL microcentrifuge tube (Eppendorf) and heated in a boiling water bath for 30 minutes. Cellular debris was pelleted by centrifugation at 14,000 rpm for 5 minutes and 500 μ L of supernatant was transferred to a clean microcentrifuge tube. Anthocyanidins were extracted with 200 μ L of *iso*-amylalcohol (IAA) and centrifuged briefly to separate the phases. The upper phase (IAA) was transferred 25 to a new tube, dried down under vacuum and resuspended in 20 μ L of IAA and a 1 μ L aliquot was spotted onto a cellulose TLC sheet (20 x 20 cm, Merck) and run for approximately 3-4 hours in Forestal solvent. The TLC plate was allowed to air dry and the anthocyanidins could be viewed under normal light.

- 28 -

c. HPLC analysis of anthocyanidins

Anthocyanins were extracted and hydrolysed by incubating approximately 0.5 g carnation petals with 1 mL of 2 M HCl at 100°C for 30 minutes. Anthocyanidins were extracted with 200 μ L of IAA. One quarter of this mixture was dried down under vacuum and resuspended 5 in 200 μ L of 50% acetonitrile and 0.5% TFA (tri-fluoro acetic acid). A 5 μ L aliquot was analysed by HPLC via gradient elution using gradient conditions of 50% B to 60% B over 10 minutes, then 60% B for 10 minutes and finally 60% B to 100% B over 5 minutes, where solvent A consisted of TFA:water (5:995) and solvent B consisted of acetonitrile:TFA:water (500:5:495). An Asahi Pac ODP-50 cartridge column (250 mm x 4.6 mm, internal diameter) 10 was used for the reversed phase chromatographic separations. The flow rate was 1 mL/minute and the temperature was 40°C. The detection of anthocyanidins was carried out using a Shimadzu SPD-M6A three-dimensional detector at 400-650 nm.

EXAMPLE 17

15

Bombardment of petals with DNA-coated microprojectiles

Particle bombardment using 1 μ m gold particles was performed essentially as described by Sanford *et al.* (1993). The biolistic Bio-Rad PDS-100 helium-driven gun with 1100 psi rupture disks was used for all bombardments. Petals were dissected from opening carnation flower buds and placed on top of a plate of MS medium (+ 0.25% gelrite) before 20 bombardment. Each plate was bombarded twice with an independently-prepared batch of DNA-coated gold particles. The plasmid DNA used for bombardment was purified using either a CsCl gradient method (Sambrook *et al.*, 1989) or a Qiagen column (Qiagen). One microgram of DNA was used per shot.

25

EXAMPLE 18

32 P-Labelling of DNA Probes

DNA fragments (50 to 100 ng) were radioactively labelled with 50 μ Ci of [α - 32 P]-dCTP using an oligolabelling kit (Bresatec). Unincorporated [α - 32 P]-dCTP was removed by chromatography on a Sephadex G-50 (Fine) column as described by Sambrook *et al.* (1989).

30

- 29 -

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

- 30 -

REFERENCES

Beld, M., Martin, C., Huits, H., Stuitje, A.R. and Gerats, A.G.M. *Plant Molecular Biology* 13: 491-502, 1989.

Bethesda Research Laboratories. BRL pUC host: *E. coli* DH5 α competent cells. *Bethesda Res. Lab. Focus.* 8(2): 9, 1986.

Brugliera, F., Holton, T.A., Stevenson, T.W., Farcy, E., Lu, C-Y. and Cornish, E.C., *The Plant Journal* 5(1): 81-92, 1994.

Bullock, W. O., Fernandez, J.M. and Short, J.M. *BioTechniques* 5: 376, 1987.

Comai, L., Moran, P. and Maslyar, D., *Plant Molecular Biology* 15: 373-381, 1990.

Ebel, J. and Hahlbrock, K., In: *The Flavonoids: Advances in Research Since 1980*. Harbourne, J.B. (ed.), Academic Press, New York, USA, 641-679, 1988.

Forkmann, G. *Plant Breeding* 106: 1-26, 1991.

Forkmann, G., and Ruhnau, B. *Z. Naturforsch.* 42c: 1146-1148, 1987.

Garfinkel, D.J. and Nester, E.W., *J.Bact.* 144: 732-743, 1980.

Geissman, T.A. and Mehlquist, G.A.L. *Genetics* 32: 410-433, 1947.

Hahlbrock, K. and Grisebach, H., *Annu. Rev. Plant Physiol.* 30: 105-130, 1979.

Hanahan, D. *J. Mol. Biol.* 166: 557, 1983.

Holton, T.A., and Graham, M.W. *Nucleic Acids Research* 19(5): 1156, 1991.

- 31 -

Holton, T.A. Isolation and characterisation of petal-specific genes from *Petunia hybrida*. PhD Thesis, University of Melbourne, 1992.

Holton, T.A., Brugliera, F. and Tanaka, Y. *The Plant Journal* 4(6): 1003-1010, 1993.

Huits, H.S.M., Gerats, A.G.M., Kreike, M.M., Mol, J.N.M. and Koes, R.E. *The Plant Journal* 6(3): 295-310, 1994.

Inoue, H., Nojima, H. and Okayama, H., *Gene* 96: 23-28, 1990.

Lassner *et al.* *Plant Molecular Biology Reporter* 7: 116-128, 1989.

Lazo, G.R., Pascal, A.S. and Ludwig, R.A. *Bio/technology* 9: 963-967, 1991.

Murashige, T. and Skoog, F. *Physiol. Plant* 15: 73-97, 1962.

Pearson, W.R. and Lipman, D.J. *Proc. Natl. Acad. Sci. (USA)* 85: 2444-2448, 1988.

Sambrook, J., Fritsch, E.F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual* (2nd edition), Cold Spring Harbor Laboratory Press, USA, 1989.

Sanford, J.C., Klein, T.M., Wolf, E.D. and Allen, N. *Journal of Particle Science Technology* 5: 27-37, 1993.

Schram, A.W., Jonsson, L.M.V. and Bennink, G.J.H., Biochemistry of flavonoid synthesis in *Petunia hybrida*. In: *Petunia* Sink, K.C. (ed.), Springer-Verlag, Berlin, Germany, pp 68-75, 1984.

Stafford, H.A., *Flavonoid Metabolism*. CRC Press, Inc. Boca Raton, Florida, USA, 1990.

- 32 -

Stich, K., Eidenberger, T., and Wurst, F. *Z. Naturforsch.* **47c**: 553-560, 1992a.

Stich, K., Eidenberger, T., Wurst, F., and Forkmann, G. *Planta* **187**: 103-108, 1992b.

Stotz, G. and Formann, G.Z. *Naturforsch* **37c**: 19-23, 1982.

Turpen, T. H. and Griffith, O. M., *BioTechniques* **4**: 11-15, 1986.

Weiss, D., van der Luit, A.H., Kroon, J.T.M., Mol, J.N.M. and Kooter, J.M. *Plant Molecular Biology* **22**: 893-897, 1993.

Wiering, H. and de Vlaming, P., Inheritance and Biochemistry of Pigments. In: *Petunia* Sink, K.C. (ed.), Springer-Verlag, Berlin, Germany, pp 49-65, 1984.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: INTERNATIONAL FLOWER DEVELOPMENTS PTY LTD

(ii) TITLE OF INVENTION: TRANSGENIC PLANTS EXHIBITING ALTERED FLOWER COLOUR AND METHODS FOR PRODUCING SAME

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: DAVIES COLLISON CAVE
(B) STREET: 1 LITTLE COLLINS STREET
(C) CITY: MELBOURNE
(D) STATE: VICTORIA
(E) COUNTRY: AUSTRALIA
(F) ZIP: 3000

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT INTERNATIONAL
(B) FILING DATE: 16-MAY-1996

(vii) PRIOR APPLICATION DATE:
(A) APPLICATION NUMBER: AU PN2988/95
(B) FILING DATE: 16-MAY-1995

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: HUGHES DR, E JOHN L
(C) REFERENCE/DOCKET NUMBER: EJH/EK

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: +61 3 9254 2777
(B) TELEFAX: +61 3 9254 2770

- 34 -

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AC(A/G)TC(A/G)GT(A/G)TGIGC(T/C)TCIACICC 23

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGGGA(A/G)GA(T/C)TA(T/C)ITITT(T/C)CA 20

CLAIMS:

1. A method of producing a plant exhibiting altered flower colour, said method comprising selecting a plant from which said first plant is derived wherein said selected plant is substantially incapable of synthesizing a DFR which acts on DHK and introducing into said selected plant one or more genetic constructs comprising nucleotide sequences encoding a F3'5'H and a DFR which is capable of acting on DHM but substantially incapable of acting on DHK.
2. A method according to Claim 1 wherein the plant is a carnation, rose, gerbera or chrysanthemum.
3. A method according to Claim 2 wherein the plant is a carnation.
4. A method according to Claim 1 or 2 or 3 wherein the selected plant is also incapable of synthesizing a F3'H.
5. A method according to Claim 1 where the nucleotide sequence encoding a DFR introduced into said selected plant is of petunia origin.
6. A method according to Claim 1 wherein the nucleotide sequence encoding a F3'5'H is from petunia, egg plant, lisianthus, gentian, pansy, china aster, anemone, grape, iris, hyacinth, delphinium or bell flower.
7. A method according to Claim 6 wherein the nucleotide sequence encoding F3'5'H is from petunia.
8. A method according to Claim 1 wherein the altered flower colour is lilac, violet, purple or blue or a shade thereof.

9. A transgenic carnation plant exhibiting the following properties:
 - (i) a substantial inability to synthesize a DFR which acts on DHK;
 - (ii) an ability to synthesize a non-indigenous DFR which is capable of acting on DHM but is substantially incapable of acting on DHK; and
 - (iii) an altered flower colour relative to a carnation plant which expresses a DFR which acts on DHK.
10. A transgenic carnation plant according to Claim 9 which also has a substantial inability to synthesize a F3'H.
11. A cut flower or a cut part containing a flower from the transgenic plant of Claim 9 or 10.
12. A transgenic plant exhibiting altered flower colour, said plant being substantially incapable of synthesizing a DFR which acts on DHK and which carries a nucleic acid molecule comprising a sequence of nucleotides which encodes a F3'5'H and a DFR which is capable of acting on DHM but substantially incapable of acting on DHK.
13. A transgenic plant according to Claim 12 wherein the plant is a carnation, rose, gerbera or chrysanthemum.
14. A transgenic plant according to Claim 13 wherein the plant is a carnation.
15. A transgenic plant according to Claim 12 or 13 or 14 wherein the plant is also substantially incapable of synthesizing a F3'H.
16. A transgenic plant according to Claim 12 wherein the nucleic acid molecule encoding the DFR is of petunia origin.

- 37 -

17. A transgenic plant according to Claim 12 wherein the nucleic acid molecule encoding the F3'5'H is from petunia, egg plant, lisianthus, gentian, pansy, chine aster, anemone, grape, iris, hyacinth, delphinium or bell flower.
18. A transgenic plant according to Claim 17 wherein the nucleic acid molecule encoding the F3'5'H is of petunia origin.
19. A transgenic plant according to Claim 12 wherein the altered flower colour is lilac, violet, purple or blue or a shade thereof.
20. A flower cut from a transgenic plant according to Claim 12 or 19 or a part of said plant.

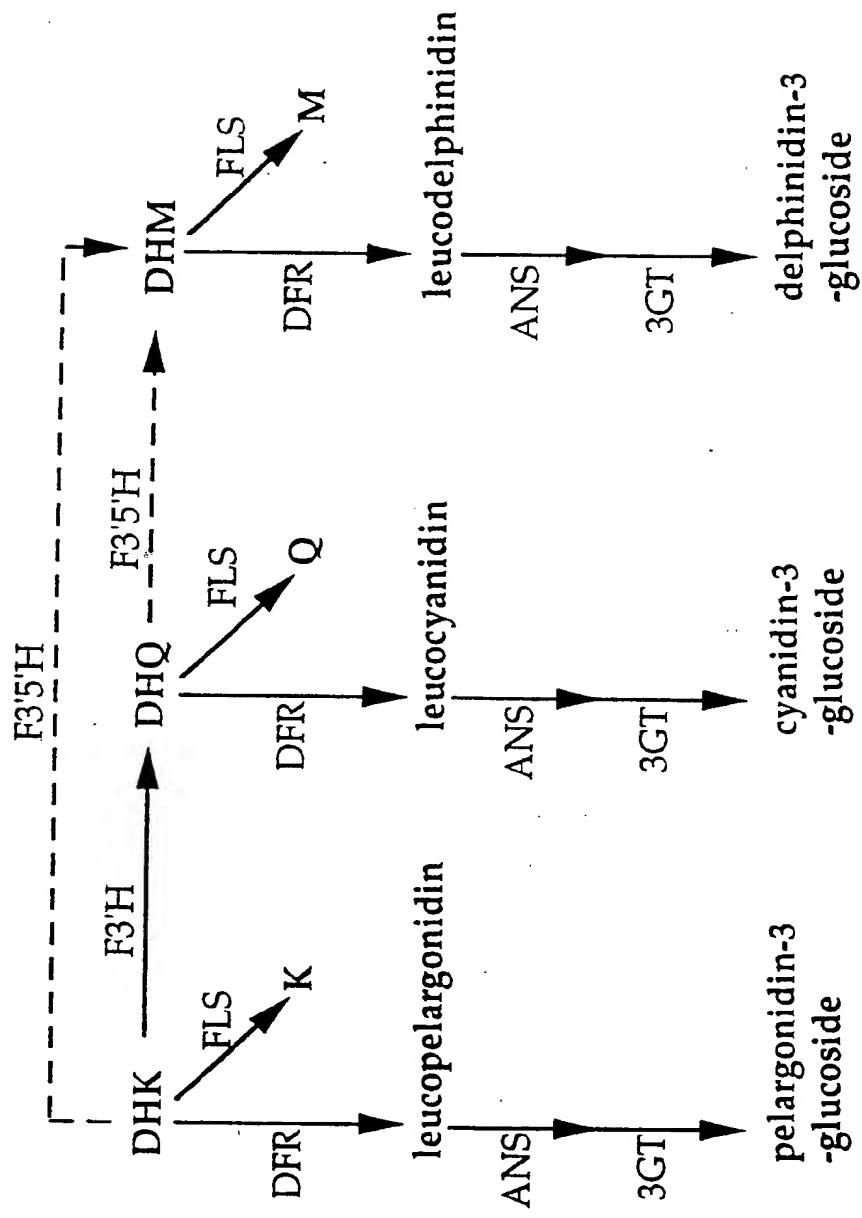


Figure 1

2/7

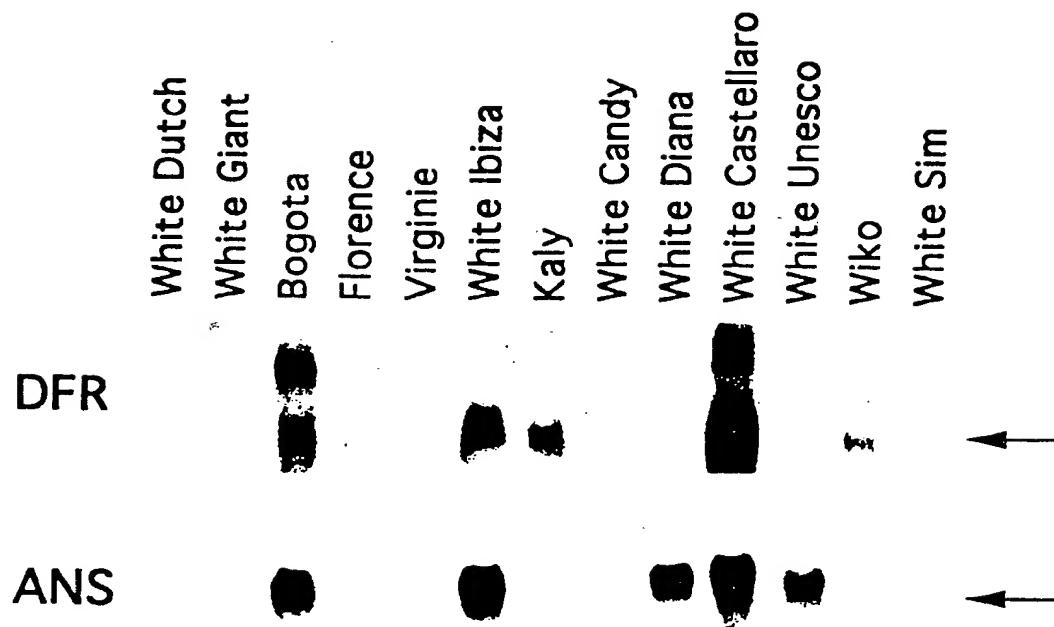


Figure 2

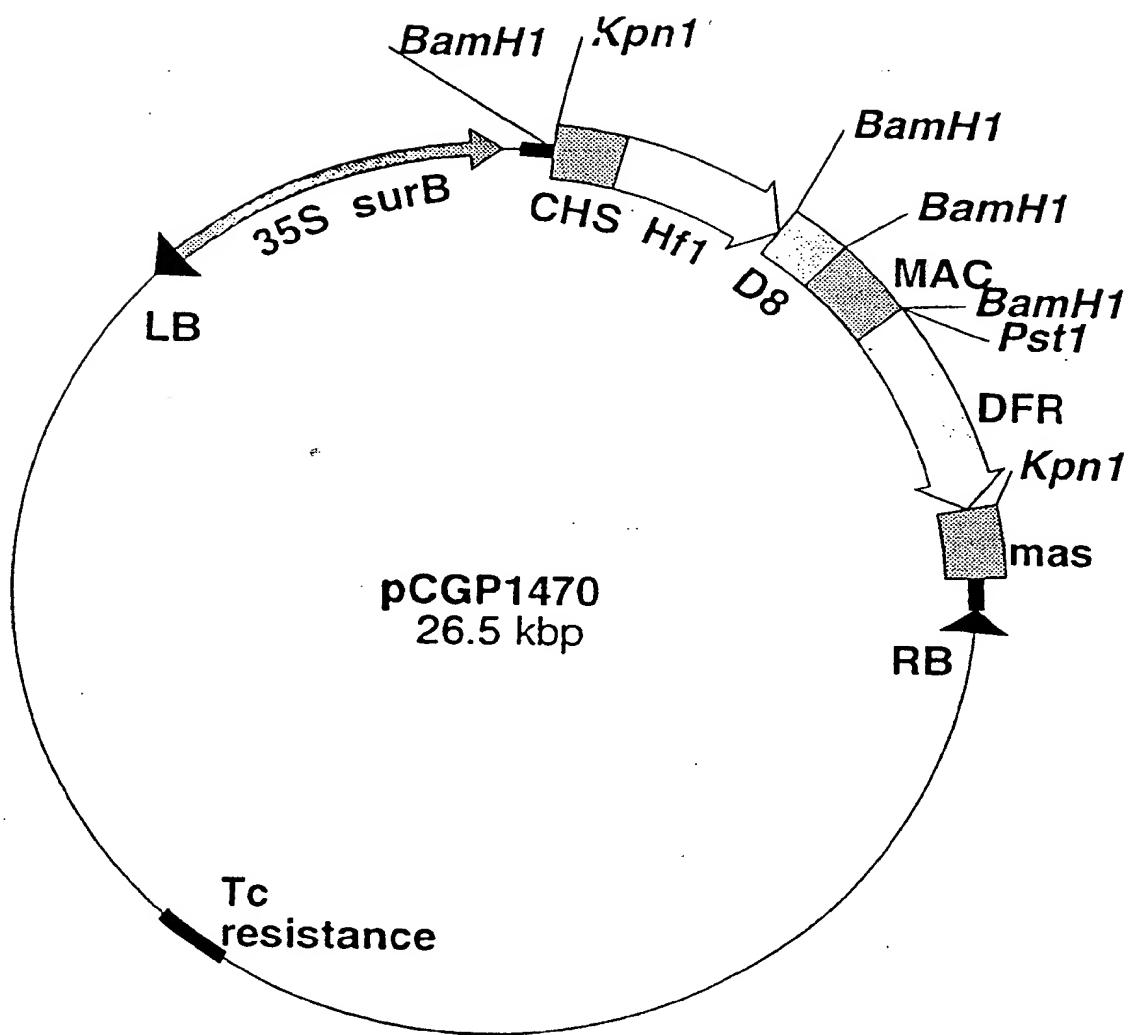


Figure 3

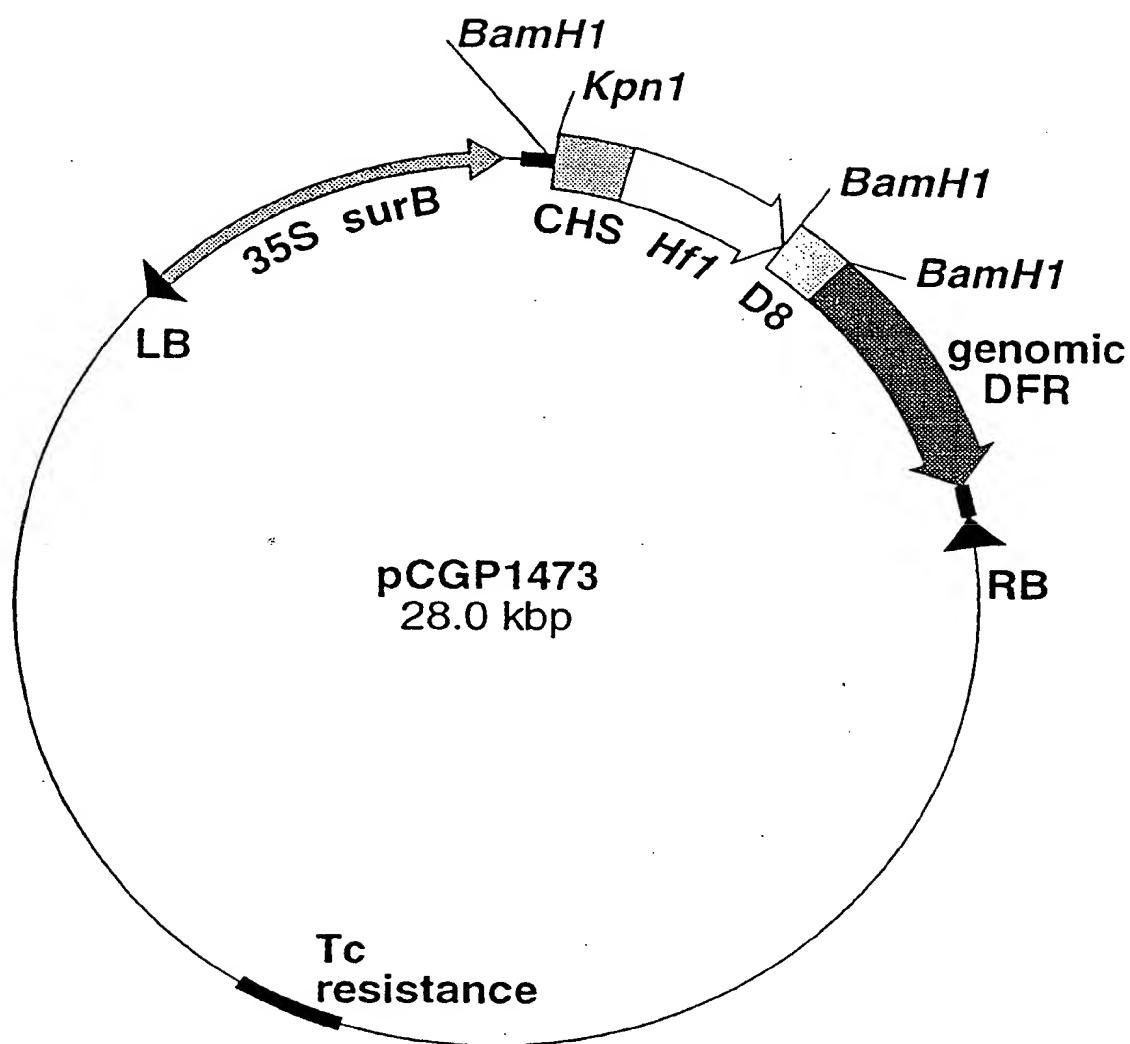


Figure 4

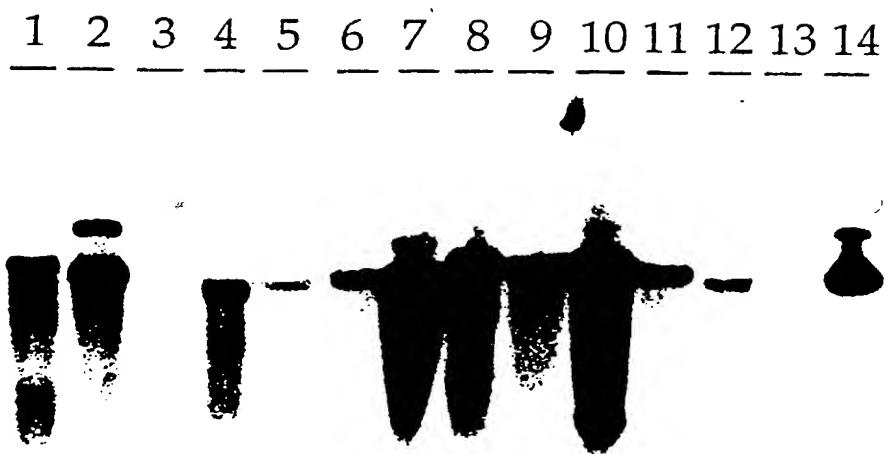


Figure 5

6/7

A



B



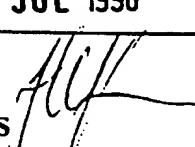
Figure 6

FIGURE 7



INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00296

| A. CLASSIFICATION OF SUBJECT MATTER | | |
|--|---|--|
| Int Cl ⁶ : C12N 15/53; A01H 5/02, 5/00 | | |
| According to International Patent Classification (IPC) or to both national classification and IPC | | |
| B. FIELDS SEARCHED | | |
| Minimum documentation searched (classification system followed by classification symbols) IPC ⁶ : C12N 15/53; A01H 5/02, 5/00 | | |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched BIOTECH | | |
| Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) DERENT - WPAT, JPAT - (DIHYDROFLAVONOL OR DIHYDROKAEMPFEROL) () REDUCTASE#, DFR, EC - 1.1.1.219 STN - CHEMICAL ABSTRACTS - AS ABOVE | | |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT | | |
| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| P,X | EUPHITICA, Volume 84 No 3, 1995, Oud, J.S.N. et al "Breeding of Transgenic orange <u>Petunia hybrida</u> varieties" pages 175-181 whole document | 1-20 |
| X | US 5410096 (MEYER et al) 25 April 1995 whole document but particularly columns 2 and 3 | 1-20 |
| <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C | | <input checked="" type="checkbox"/> See patent family annex |
| * Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed | | |
| "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family | | |
| Date of the actual completion of the international search 18 July 1996 | | Date of mailing of the international search report 29 JUL 1996 |
| Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929 | | Authorized officer JESSICA WYERS Telephone No.: (06) 283 2624  |

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00296

| C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|--|---|-----------------------|
| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| X | BIOTECHNOL. GENET. ENG. REV. Volume 12, December 1994, Elomaa, P and Holton, T. "Modification of Flower Colour using Genetic Engineering" pages 63-88 whole document but particularly pages 79-81 | 1-20 |
| A | PLANTA, Volume 187, No. 1, 1992 Stich, K. et al "Enzymatic conversion of dihydroflavonols to flavan-3,4-diols using flower extracts of <u>Dianthus caryophyllus</u> L. (carnation)" pages 103-108 whole document | 1-20 |
| A | Z. NATURFORSCH, C: BIOSCI., Volume 42, 1987, Forkmann, G. and Ruhnau, B. "Distinct Substrate Specificity of Dihydroflavonol-4-Reductase from Flowers of <u>Petunia hybrida</u> " pages 1146-1148 whole document | 1-20 |

INTERNATIONAL SEARCH REPORT**Information on patent family members**

International Application No.
PCT/AU 96/00296

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent Document Cited in Search Report | | | | Patent Family Member | | | |
|--|---------|----|---------|----------------------|---------|----|---------|
| US | 5410096 | DE | 3738657 | DE | 3885061 | EP | 316797 |
| | | ES | 2059472 | IL | 88307 | JP | 2002305 |

END OF ANNEX